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SOX9 is up-regulated by the transient expression of SRY specifically in Sertoli cell precursors

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Abstract

The Y chromosome gene *Sry* encodes a transcription factor required to initiate testis development. The related autosomal gene *Sox9* is up-regulated shortly after the onset of *Sry* transcription and is thought essential for the differentiation of Sertoli cells. The lineage that gives rise to Sertoli cells has its origins within the coelomic epithelium (CE) of the genital ridge, but from cells also able to give rise to an interstitial cell type. It was not known at what point SRY acts in the derivation of this lineage or how the two genes interact. To investigate the identity of the cells expressing *Sry*, we designed two transgenes driven by the *Sry* promoter: one gives expression of a stable reporter, human placental alkaline phosphatase (hPLAP), while the second gives expression of a functional Myc-epitope tagged SRY protein (SRY^{MYC}). Taking advantage of lasting hPLAP activity after transcription of the reporter gene has ceased, we could show that Sry^{hPLAP} was expressed exclusively in all cells fated to become Sertoli cells. SRY^{MYC}-single-positive cells were first observed in the gonad and not in the CE. Subsequently, they became SRY^{MYC}/SOX9-double-positive, but only for a few hours before turning into SOX9-single-positive cells. After the coelomic epithelial cells migrate into the gonad, there is first a decision to become interstitial or supporting cells, and then the transient expression of SRY in the latter determines their fate as Sertoli cells by up-regulating *Sox9*.

Keywords: Sry; Sox9; Testis development; Sertoli cell; Transgenic mice

Introduction

In mammals, the presence of the Y chromosome acts as a dominant male determinant. *Sry* was identified as the only gene on the Y chromosome required to initiate male development because mice or humans carrying deletions or point mutations of the *Sry/SRY* gene show an XY female sex-reversed phenotype (Gubbay et al., 1990; Sinclair et al., 1990), and a 14-kb genomic fragment of the *Sry* gene (Fig. 1A) induced male sex reversal when it was introduced into XX mice as a transgene (Koopman et al., 1991). *Sry* encodes a transcription factor with an HMG box DNA-binding domain, and no direct target gene(s) of SRY have yet been identified despite being discovered some 13 years ago. The autosomal gene *Sox9*, which encodes a related HMG box-containing factor, is up-regulated specifically in XY gonads shortly after the onset of *Sry* transcription and is thought essential for the differentiation of Sertoli cells (Kent et al., 1996; Morais da Silva et al., 1996). The lineage that gives rise to Sertoli cells has its origins within the coelomic epithelium (CE) of the genital ridge, but from cells also able to give rise to an interstitial cell type (Karl and Capel, 1998). It was not known at what point *Sry* acts in the derivation of this lineage or how the two genes interact, because *Sry* transcripts are no longer present in the testis once it can be

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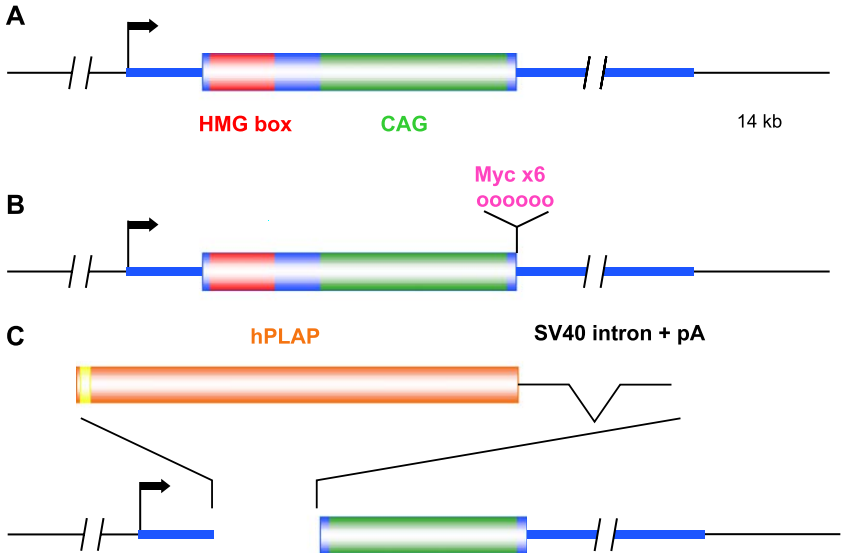

Fig. 1. Schematic representation of *Sry* transgenes. (A) 14-kb *Sry* genomic fragment described previously, which is sufficient for sex reversal (Koopman et al., 1991). (B) Sry^{Myc} was constructed by insertion of six copies of the Myc-epitope tag (pink circles) as an in-frame fusion immediately before the stop codon. (C) Sry^{hPLAP} was constructed by replacing the 5′ half of the coding region with the human placental alkaline phosphatase gene (*hPLAP*) (orange box) with SV40 splice and polyadenylation signals. *hPLAP* contains a signal peptide sequence (yellow box) on its 5′ end, which allows it to be localized to the plasma membrane of cells. The untranslated regions and the coding region of *Sry* are indicated by blue bars and the blue box, respectively. The red box encodes the HMG box DNA-binding domain. The green box shows a CAG-rich sequence which encodes a glutamine-rich region.

recognized (Hacker et al., 1995). To investigate the identity of the cells expressing *Sry*, we designed two transgenes driven by the *Sry* promoter: one gives expression of a stable reporter, human placental alkaline phosphatase (hPLAP), while the second gives expression of a functional Myc-epitope tagged SRY protein (SRY^{MYC}). Taking advantage of lasting hPLAP activity after transcription of the reporter gene has ceased, we could show that Sry^{hPLAP} was expressed exclusively in all cells fated to become Sertoli cells. SRY^{MYC}-single-positive cells were first observed in the gonad and not in the CE. Subsequently, they became SRY^{MYC}/SOX9-double-positive, but only for a few hours before turning into SOX9-single-positive cells. Moreover, we found that *Sox9* expression is altered in *Sry* mutants, confirming that *Sox9* is genetically downstream of *Sry*. Our data suggest that there is first a decision to become interstitial or supporting cells after the coelomic epithelial cells migrate into the gonad, and then the transient expression of SRY in the latter determines their fate as Sertoli cells by up-regulating *Sox9*.

Materials and methods

Construction of plasmids and generating transgenic mice

To generate the Sry^{Myc} construct, we introduced a new *Xho*I site at the stop codon, position 9490 on the 14-kb 741 genomic clone (Koopman et al., 1991). The *Xho*I site was blunt-ended, and a *Bam*HI-*Stu*I fragment containing six copies of c-Myc tag derived from pCS2 + MT (Roth et al., 1991) was inserted also after being blunt-ended. Sry^{hPLAP} was generated by replacing the *Eco*RV (8224)–*Eco*RV (8712) fragment with the human placental alkaline phosphatase gene (Kam et al., 1985) and SV40 small t gene. Transgenes were released from plasmids by *Sal*I digestion and injected into pronuclei of fertilized eggs from F1 (C57BL/10 × CBA) mice. The injected embryos were transferred into oviducts of pseudopregnant F1 recipients. Transgenic mice were identified by PCR using 5′-ATGGAGAGCTTGGGCGACCTC-3′ and 5′-AATCATAGCAAGGGGGAGTGTTG-3′ for Sry^{Myc}, and 5′-AGAGCGACATATGGGAAGCG-3′ and 5′-AAGTTTTGACTTCTGTATCTGTCTG-3′ for Sry^{hPLAP}. Sex-reversed XY^{Tdym1} females (Lovell-Badge and Robertson, 1990) and $XY^{Tdym1}Sry/+$ males were generated by crossing between XY^{Tdym1} females and XY*Sry*/+ males (Mahadevaiah et al., 1998).

In situ hybridization, immunohistochemistry, and alkaline phosphatase staining

In situ hybridization of *Sox9* was described by Morais da Silva et al. (1996). The full-length hPLAP cDNA (Kam et al., 1985) was used as a probe for in situ hybridization of the Sry^{hPLAP} transgene. To detect SRY^{MYC} expression, gonads were fixed with MEMFA (4% formaldehyde, 1 mM $MgCl_2$, 2 mM EGTA, 100 mM MOPS, pH 7.4) for 30 min at 4°C and subsequently bleached with 3% H_2O_2, 0.1% Triton X-100 in PBS for 30 min at room temperature and incubated in PBS containing 1.5% blocking reagent (Boehringer Mannheim), 0.1% Triton X-100 for 60 min. As a primary

antibody, we used anti-c-Myc rabbit polyclonal IgG (A-14, Santa Cruz Biotechnologies, Inc.) in 1:200 dilution and incubated overnight at 4°C. After washing and blocking, anti-rabbit IgG conjugated to peroxidase (Sigma) was added and incubated overnight at 4°C. Peroxidase activities were detected in 1 mg/ml diaminobenzidine tetrahydrochloride, 0.03% H_2O_2, 0.1% Triton X-100, PBS. For cryosections, after incubation with 30% sucrose in PBS overnight, gonads were embedded in O.C.T. compound and sectioned at 10 μm. After incubation with anti-c-Myc rabbit polyclonal IgG followed by 1:350 diluted anti-rabbit IgG conjugated to Cy3 (Amersham Pharmacia Biotech), unbound IgGs were inactivated by microwaves for 5 min. Sections were incubated in 1:1000 diluted anti-SOX9 rabbit antiserum (Morais da Silva et al., 1996) and then 1:200 diluted anti-rabbit IgG conjugated to Alexa Fluor 488 (Molecular Probes). To detect alkaline phosphatase activities, we followed the method described by Ginsburg et al. (1990) for primordial germ cells and by Sharpe et al. (1998) for the Sry^{hPLAP} transgene.

Results and discussion

MYC epitope-tagged SRY causes sex reversal

To investigate *Sry* action, it is important to know when and where SRY protein is expressed. However, attempts to make an antibody against mouse SRY that would allow immunohistochemistry studies in vivo have failed, while antibodies raised against human SRY do not cross-react with the mouse protein because their amino acid sequences are too different. To circumvent this problem, six copies of the c-Myc epitope tag were inserted just before the mouse SRY stop codon in the context of the 14-kb genomic fragment of the *Sry* locus (Fig. 1B). The Sry^{Myc} transgene was injected into fertilized eggs and four independent lines, A–C and E, were generated from XY male founders (see Supplemental Table 1). A fifth founder, D, was an XX female, so it was assumed that the transgene was not sufficiently active to be useful. Male founders were mated with wild-type females to test if the transgene led to XX male sex reversal. We observed 100% sex reversal in three lines, A, B, and E, and 33% in line C (Fig. 2A). Line C expresses Sry^{Myc} at a lower level than in the other lines (data not shown), consistent with previous studies showing that 'partial' sex reversal is a consequence of transgene expression levels below a critical threshold of about 50% compared to the endogenous *Sry* gene (Burgoyne et al., 2001; Capel et al., 1993; Lovell-Badge, 1993; Swain et al., 1998). In the three lines showing complete sex reversal, adult testes of XX Sry^{Myc} mice were smaller than those of XY Sry^{Myc} mice (Figs. 2B, C) or wild-type males (data not shown) because the presence of two X chromosomes is incompatible with spermatogenesis (Lyon et al., 1981). In embryonic testes, however, testis cord formation was apparent in XX gonads and showed no delay in comparison with XY gonads of the same development stage as judged by tail somite number (data not shown). These data show that the MYC epitope tag does not interfere with SRY function in vivo.

Expression of SRY^{MYC} protein during gonadal development

Studies using RNAse protection assays, RT–PCR, and in situ hybridization all show the presence of mouse *Sry* transcripts, specifically within the developing genital ridge, during a fairly narrow time window (Bullejos and Koopman, 2001; Hacker et al., 1995; Jeske et al., 1995; Swain et al., 1998). This starts at the 11–12-tail somite stage (ts), corresponding on average to a few hours after 10.5 days postcoitum (dpc), reaches a peak at 18 ts (11.5 dpc), and declines towards 27 ts (shortly before 12.5 dpc). However, it has been difficult to unambiguously identify the cell type in which these transcripts appear. To investigate the expression of the Sry^{Myc} transgene, we used an anti-MYC antibody and performed immunostaining during gonadal development, focusing on lines C and E. At 8 ts, (10.5 dpc on average), there were no cells positive for SRY^{MYC}. The protein was first detected at 12 ts in a few cells located below the epithelium in the central region of both XY and XX gonads of lines C (Fig. 5A) and E (data not shown). At 18 ts, SRY^{MYC} expression was seen in cells scattered throughout the gonads, again in both lines C (Figs. 2D, E) and E (Figs. 2F, G). In the testes of XY and XX sex-reversed mice, the expression was absent at 30 ts (corresponding on average to 12.5 dpc), (Fig. 2H). Double labeling for alkaline phosphatase activity showed that there was no expression of SRY^{MYC} in germ cells (see Supplemental Fig. 1), which is in contrast to the situation in humans (Salas-Cortés et al., 1999). These expression data agree with previous studies looking at *Sry* transcripts (Bullejos and Koopman, 2001; Hacker et al., 1995; Jeske et al., 1995; Swain et al., 1998), with very little delay in the first appearance and final loss of the protein compared to mRNA. This is consistent with efficient translation and with the protein having a short half-life. There was no qualitative difference between lines C and E, with similar numbers of cells expressing SRY^{MYC} at all stages examined. However, in line C, where the transgene often failed to sex-reverse XX embryos due to its lower level of expression, SRY^{MYC} was still present in ovaries at 30 ts (Fig. 2I) and subsequently up to 14 dpc (data not shown). These results suggest that there is a negative feedback mechanism for turning off *Sry* transcription, but only if the levels of SRY protein reach above the critical threshold required to initiate testis development. This is consistent with previous findings from Lee and Taketo (1994) where expression of *Sry* was prolonged in sex-reversed ovaries/ovotestes in embryos carrying a weak allele

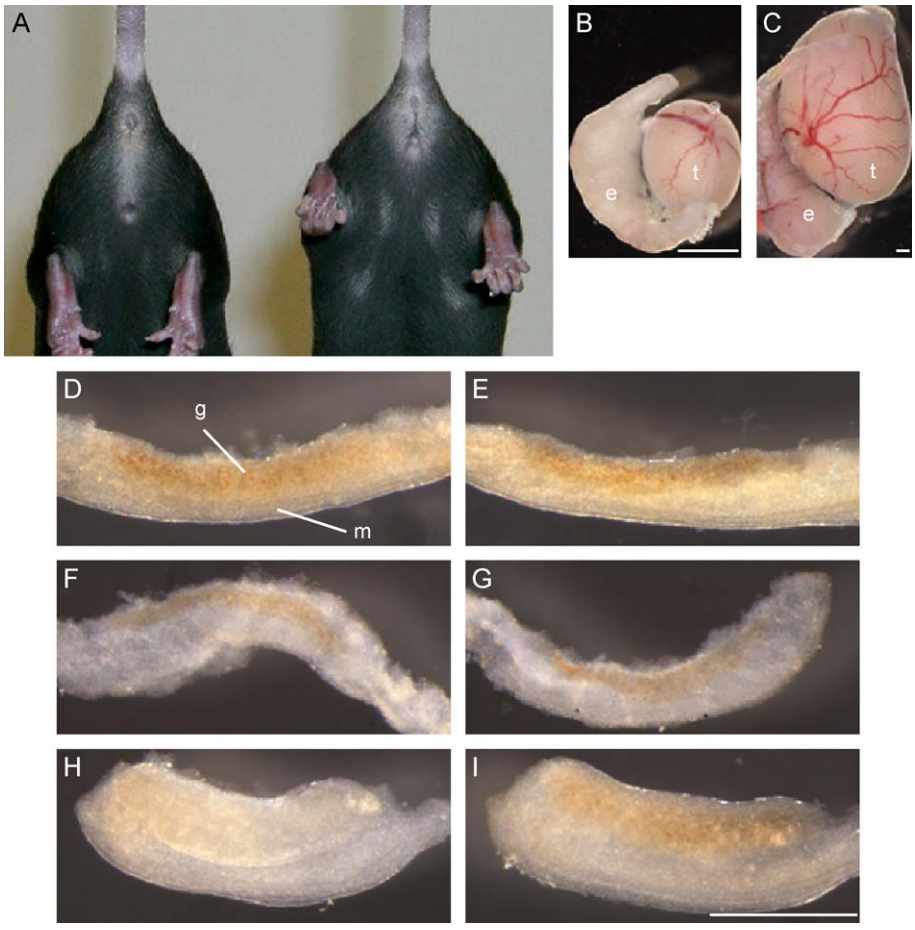

Fig. 2. Sex-reversed phenotype of *Sry*^{Myc} transgenic mice and the expression of SRY^{MYC} protein. (A–C) Eleven-week-old litters derived from line E were analyzed for sex-reversed phenotype. External genitalia with male character was seen in an XX *Sry*^{Myc} transgenic mouse (left) comparing with a wild-type XX female (right) (A). XX *Sry*^{Myc} testis (B) were smaller than XY *Sry*^{Myc} testis (C) because of lack of sperm in XX testes. (D–I) The embryonic gonads were dissected from the incompletely penetrant line C (D, E and H, I) and a completely penetrant line E (F, G). At 18 ts, expression was observed throughout both XY (D) and XX (E) indifferent gonads in line C. No significant differences were seen in either XY (F) or XX (G) indifferent gonads in line E. At 30 ts, the expression was barely detectable in XY testes (H). In the case of non-sex-reversed XX *Sry*^{Myc} females in line C, expression was maintained in the ovaries (I). Scale bars, 1 mm. Abbreviations: e indicates epididymis; g, gonad; m, mesonephros; t, testis.

of *Sry* on a C57BL6/J background. Perhaps, in both these cases, SRY fails to reach above a particular threshold.

SRY^{hPLAP} *is expressed exclusively in the Sertoli cell lineage*

From direct cell lineage studies using DiI, it was found that descendents of cells within the CE can migrate into the gonads in both sexes, and it was possible to follow their fate in the testes (Karl and Capel, 1998). At early stages (before 18 ts), the labeled cells have the potential to give at least three different cell types: Sertoli cells within the testis cords, an interstitial cell type of unknown identity, and tunica cells when they remain in the CE. At later stages (post-18 ts), cells within the CE appear to have lost the ability to contribute to Sertoli cells, although it is possible that they are physically excluded from the cords and, being in the wrong environment, differentiate into the interstitial cell type or die. Since SRY^{MYC} is no longer found in the testis at a time when the various somatic cell types can be identified, the question remains as to whether expression of the gene is restricted to the precursors of Sertoli cells, or whether it can also be found in other lineages. To overcome this problem, we replaced a part of the coding region of *Sry* with that of the human placenta alkaline phosphatase (hPLAP) gene, also in the context of the 14-kb genomic fragment of Sry, to give *Sry*^{hPLAP}, (Fig. 1C). The human enzyme is stable, even at high temperature, and its activity is known to persist for some time after transcription is turned off. This should enable us to trace the cell lineage in which the transgene was expressed. Three out of three transgenic mouse lines showed expression of the *hPLAP* transcript specifically within the genital ridge at 18 ts, but not at 30 ts. This was shown by in situ hybridization (Figs. 3A–D) and by RT–PCR (data not shown), confirming that expression of the transgene mirrored that of the endogenous gene. In contrast, hPLAP activity from the transgene, which was also readily detected at 18 ts, was retained strongly at 30 ts in XY embryos (Fig. 3F). It should be noted that endogenous alkaline phospha-

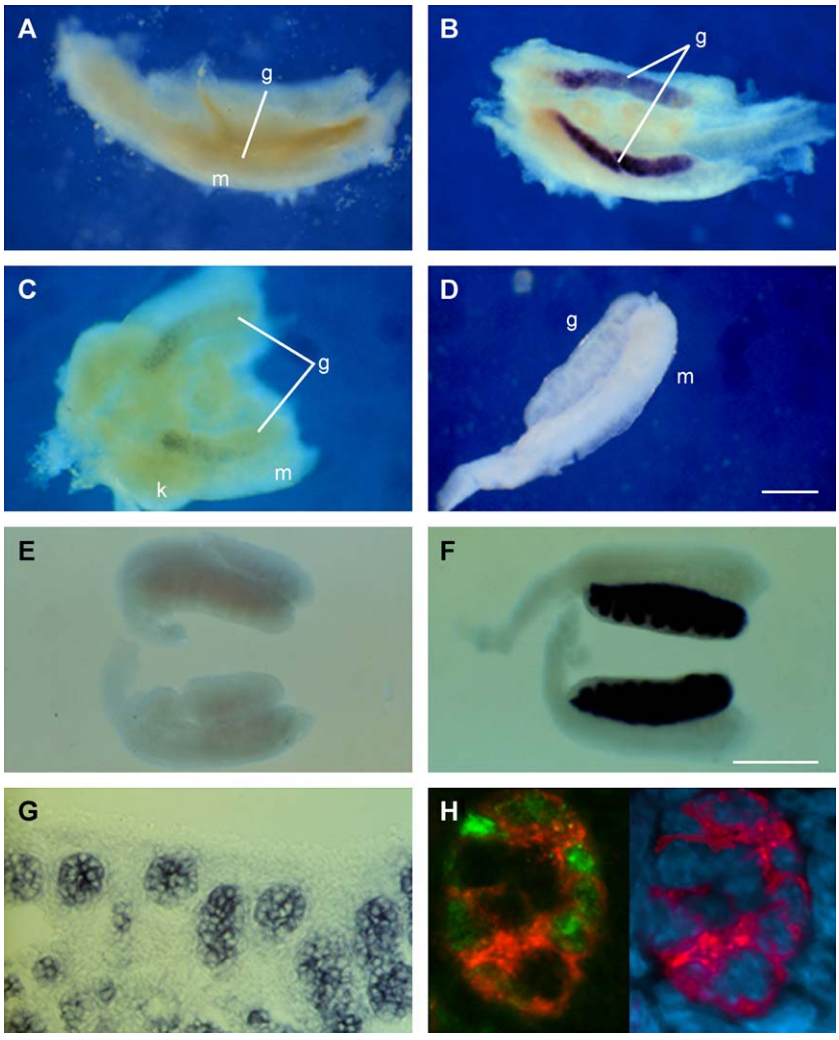

Fig. 3. Sertoli cell lineage-specific expression of the Sry^{hPLAP} transgene. (A–D) In situ hybridization with Sry^{hPLAP} transgene showed that the *hPLAP* transcript is strongly expressed in XY gonads from the transgenic mice (B) but not from wild-type mice (A) at 18 ts. The expression level of the transcript decreases in XY gonads at 24 ts (C) and is hardly detectable at 30 ts (D). (E–G) However, enzyme activity from Sry^{hPLAP} transgene is prominent throughout testis cords of an XY Sry^{hPLAP} male at 30 ts (F). Before the detection of hPLAP activity, endogenous alkaline phosphatase activity is inactivated by heat treatment at 65°C, so shown by the absence of staining in testes of wild-type XY mice at this stage (E). The gonad shown in (F) was sectioned to confirm that Sry^{hPLAP} activity was detected at the plasma membrane of cells only in the testis cords (G). (H) A section of a XY Sry^{hPLAP} testis cord double-stained for SOX9 protein (green signal in nuclei) and Sry^{hPLAP} activities (red signal at plasma membrane) showed that they colocalize in the same cells (left). The same section was viewed with DAPI staining (blue) and Sry^{hPLAP} activity (right). Scale bars, 1 mm. Abbreviations: g indicates gonad; k, kidney; m, mesonephros.

tase activity, including the high levels in PGCs, was completely inactivated by the heat treatment used in the protocol (Fig. 3E).

At 30 ts, the structure of the testis and many of the cell types can be recognized morphologically. SRY^{hPLAP} was detected only within testis cords and not in interstitial cells of XY transgenic embryos (Fig. 3G). The cords contain PGCs and Sertoli cells. Given that the PGCs did not express Sry^{Myc} (see above), they are also unlikely to express Sry^{hPLAP}. In the context of the gonad, SOX9 is a very good marker of Sertoli cells, at least after 18 ts when they can also be identified by morphological criteria (Kent et al., 1996; Morais da Silva et al., 1996). Using specific antibodies to SOX9, we find that all cells positive for hPLAP activity, which is localized to the plasma membrane, also show nuclear staining for SOX9 and vice versa (Fig. 3H). SRY therefore acts uniquely within the supporting cell lineage to direct their fate to that of Sertoli cells, and in a normal situation, there is no recruitment of cells that have not expressed *Sry* into this lineage. This is in contrast to the results of Burgoyne et al. (Burgoyne et al., 1988; Palmer and Burgoyne, 1991) and Patek et al. (1991), who found that some XX cells could differentiate as Sertoli cells in XX-XY chimaeras. It is possible that the XX cells acquire a Sertoli cell phenotype relatively late in these cases and/or that the presence of cells of the correct lineage can be recruited to differentiate as Sertoli cells even if they do not contain *Sry*, whereas in our case, all cells contain *Sry* and the Sry^{hPLAP} transgene.

Albrecht and Eicher (2001) also used a transgenic approach to explore *Sry* expression and gonadal cell

lineages. In this study, a 7762-bp 5′ flanking region from *Sry* was used to drive expression of an *EGFP* reporter gene. However, Sry^{egfp} transcription was not down-regulated appropriately by 12.5 dpc (30 ts), and transcripts were still present at 15.5 dpc and even postnatally at P1 and P28, while EGFP could be detected as late as 15.5 dpc. It was concluded that *Sry* is expressed in the supporting cell lineage, and our data agree with this, but continual transcription strictly precludes use of this transgene as a lineage marker. A comparison of their results with ours indicates that the region 3′ to *Sry* must contain essential information for its correct regulation. This is consistent with our own unpublished data, where 5′ sequences alone are unable to drive correct expression of reporter genes, including *hPLAP* and with data obtained by Jo Bowles and Peter Koopman (personal communication), who were able to delete all sequences 5′ to the transcriptional start site and still efficiently obtain XX male sex reversal.

Sox9 is genetically downstream of Sry

Sox9 is expressed at very low levels in the genital ridge of both sexes at 10.5 dpc. By 11.5 dpc, however, *Sox9* expression has become strongly up-regulated in the male gonad (Fig. 4A), while it is down-regulated in the female (Fig. 4B) (Kent et al., 1996; Morais da Silva et al., 1996). *Sox9* expression appears to be abolished in mice lacking the +KTS isoform of WT1, equivalent to Frasier syndrome in humans (Hammes et al., 2001), and also in $Sf\text{-}1^{-/-}$ mice (Swain, Guioli, and L-B, unpublished data). Therefore, both WT1 and SF-1 may well be required to initiate *Sox9* expression. Since the up-regulation of *Sox9* closely follows the onset of *Sry* expression, and since it appears to have a critical role in sex determination, *Sox9* has been thought to be a good candidate for a direct target gene of SRY (Lovell-Badge et al., 2002). Further genetic evidence that *Sox9* expression depends on SRY rather than an on another Y-linked gene or an X chromosome dosage effect was obtained by finding that its expression was absent from genital ridges of XY^{Tdym1} embryos (Lovell-Badge and Robertson, 1990; Mahadevaiah et al., 1998), which lack *Sry* (Fig. 4C) but present at a high level in those of XX embryos carrying a sex-reversing *Sry* transgene (Fig. 4D).

There is considerable evidence from both loss and gain of function mutations that *Sox9* is required for male development. Haploinsufficiency of the *SOX9* gene in humans causes the skeletal malformation syndrome, campomelic dysplasia with approximately 75% of XY patients also showing male-to-female sex reversal (Foster et al., 1994; Kwok et al., 1995; Preiss et al., 2001; Wagner et al., 1994), furthermore, homozygous loss of *Sox9* in mice leads to XY ovary development (Chaboissier et al., 2004). In contrast, an XX individual with a duplication of 17q23–24 containing *SOX9* showed female-to-male sex reversal (Huang et al., 1999). In mice, the insertion of a tyrosinase minigene 1 Mb upstream of *Sox9* (and an associated 100-kb deletion) leads to constitutive activation of *Sox9* within the gonad. Consequently, all these transgenic mice, named *Odsex* (*Ods*), can develop as males irrespective of the presence of *Sry* (Bishop et al., 2000). Moreover, it was reported recently

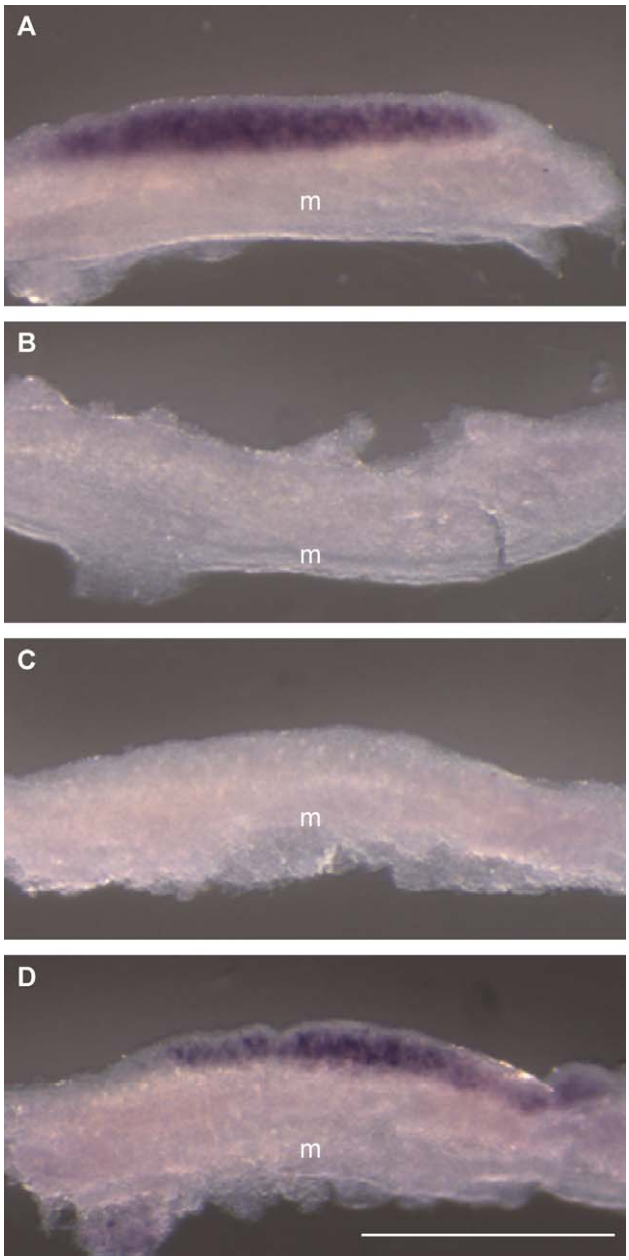

Fig. 4. *Sox9* expression is altered in indifferent gonads of *Sry* mutants. XY mice carrying the Tdy^{m1} mutation are fertile sex-reversed females due to an 11-kb deletion of the Y chromosome encompassing the *Sry* gene. This sex reversal can be 'rescued' by breeding in an autosomal *Sry* transgene, which gives fertile male mice that are $XY^{Tdym1}Sry/+$. Crosses between $XY^{Tdym1}Sry/+$ males and wild-type XX females gives rise to wild-type XY males and XX females and to XY^{Tdym1} null mutant female and XX*Sry*/+ transgenic male. (A–D) In situ hybridization for 18 ts indifferent gonads shows that *Sox9* is expressed in wild-type XY male (A) but not XX female (B). In XY^{Tdym1} female, *Sox9* expression is totally abolished (C). On the other hand, it is induced in XX*Sry*/+ male (D). Scale bars, 1 mm. Abbreviation: m indicates mesonephros.

that misexpression of *Sox9* driven by the *WT1* promoter gave rise to female-to-male sex reversal in transgenic mice (Vidal et al., 2001). All these findings suggest that *Sox9* is the only critical gene downstream of SRY, which adds weight to the suggestion that it is a direct target.

SRY^{MYC} and SOX9 are transiently colocalized in Sertoli cell precursors

If SRY up-regulates *Sox9* expression directly, they would need to be coexpressed earlier than 30 ts, within the Sertoli cell lineage. To test this, we used antibodies against both SOX9 and the MYC epitope on sections of genital ridges of *Sry^{Myc}* transgenic embryos. Neither protein was detectable at 11 ts (data not shown). A few SRY^{MYC}-positive cells were first found at 12 ts, but SOX9 was still undetectable at this stage (Fig. 5A). At 18 ts, there were more SRY^{MYC}-positive cells, but most of these were now also positive for SOX9. However, many cells were seen at this stage that were positive for SOX9 alone (Fig. 5B). Subsequently, the proportion of SRY^{MYC}-positive cells decreased, and most cells in the gonad became SOX9-positive by 25 ts (Fig. 5C). A rough estimate of the relative proportion of cells that were single or double-positive for the two proteins was obtained by comparing these cells with the total number of cells in the sections shown in Fig. 5. Thus, at 18 ts, approximately 4% of cells were positive for SRY alone, 14% were double-positive, and 18% just expressed SOX9. At 25 ts, the relative proportions being 2% SRY^{MYC}-single-positive, 6% double-positive, and 50% SOX9-single-positive. To be consistent with our conclusions reached above, that all SRY-positive cells become Sertoli cells, we assume that the SOX9-single-positive cells were the descendents of cells that had once been SRY^{MYC}-positive. This implies that the lifetime of SRY^{MYC} protein is very short in each cell and probably substantially less than the 14-h time difference between 12 and 18 ts embryos. As we see SOX9-single-positive cells by 15 ts, this suggests that SRY can be present for no more than 7 h (i.e., the time difference between 12 and 15 ts).

Human SRY has been reported to be present in the cytoplasm of some cells in the early genital ridge (Harley, 2002) and also reported to be expressed in a nuclear speckle pattern implicating a role in pre-mRNA splicing (Ohe et al., 2002). However, we could not detect SRY^{MYC} in either the cytoplasm or nuclear speckles at any stage (Figs. 5D, E). With respect to SOX9, some of this present data contrast with our earlier expression studies showing that *Sox9*

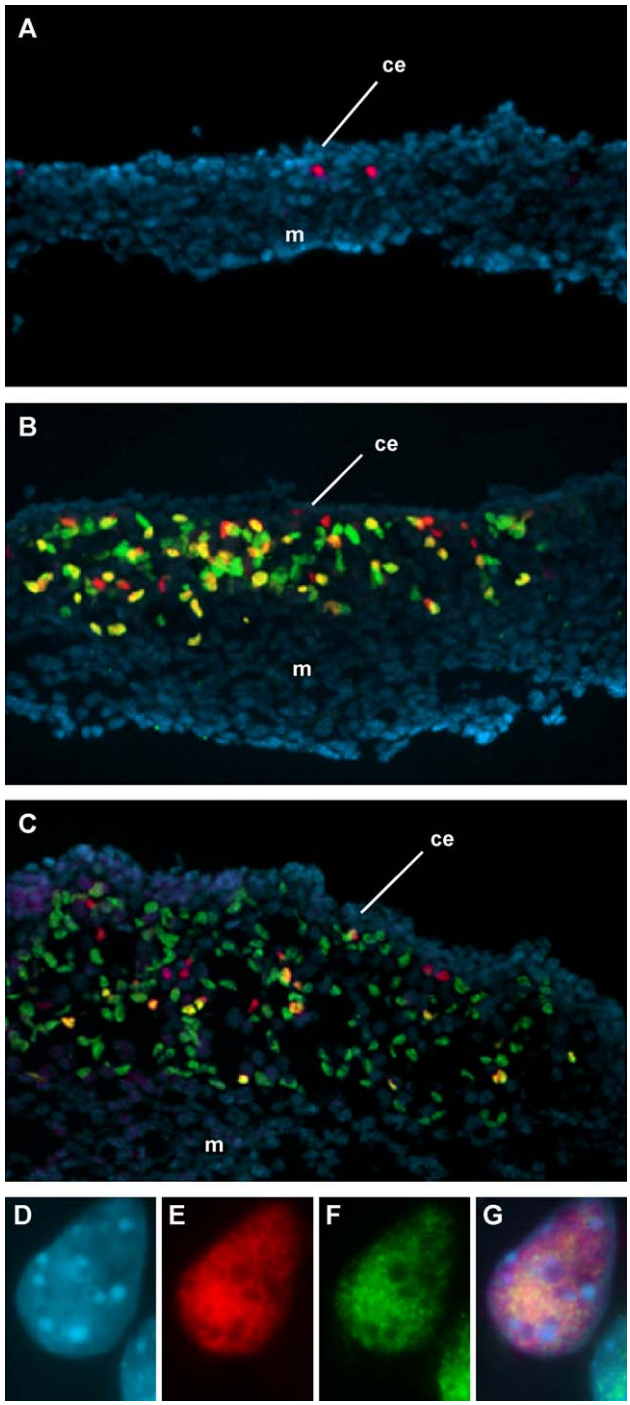

Fig. 5. Colocalisation of SRY^{MYC} and SOX9 proteins during male gonadal development. The gonads derived from XY*Sry^{Myc}* males were immunostained for SRY^{MYC} (red) and SOX9 (green) with DAPI (blue). (A) At 12 ts, a few SRY^{MYC}-positive cells (red) were observed directly underneath, but not at the CE. SOX9-positive cells were not detected in these conditions. (B) At 18 ts, SRY^{MYC}-positive cells were not only directly underneath the CE but also further inside the gonads. Approximately 78% of SRY^{MYC}-positive cells were SOX9-positive as well (yellow) and 56% of SOX9-positive cells were already SOX9-single-positive at this stage. (C) At 25 ts, the population of SRY^{MYC}-positive cells decreased while SOX9-positive cells occupied major parts of the gonad. SRY^{MYC}-single-positive cells, presumably newly migrated into the gonad, were still observed. (D–G) A merged image of a double-positive cell (G) at 18 ts showing SRY^{MYC} (E) and SOX9 (F) colocalized within the nucleus. DAPI strongly stains heterochromatin regions (D). Neither SRY^{MYC} nor SOX9 were expressed in these regions. The same colocalization pattern was observed from 14 to 25 ts (data not shown). Abbreviations: ce indicates coelomic epithelium; m, mesonephros.

transcripts are present at a low level in both XY and XX gonads before 12 ts, before the initiation of *Sry* expression in males (Morais da Silva et al., 1996). Moreover, it has been reported that SOX9 protein is cytoplasmic in the very early genital ridges of both sexes in both mouse and human (Barbara et al., 2000; Morais da Silva et al., 1996), but we were unable to detect SOX9 in the cytoplasm at any stage because the first time we detect SOX9 expression is in cells that are also positive for SRY^{MYC} (Figs. 5E–G). In the present study, C57BL/10 × CBA hybrid mice were used instead of the outbred strain (Parkes) looked at previously, and the expression level of *Sox9* may be background-dependent. We may also have lost sensitivity using both a different aliquot of anti-SOX9 antibody and through double staining for the MYC-epitope.

Conclusions

In the experiments reported here, we show precisely when and which cell types SRY is active, and for how long. Several cellular events that depend on SRY activity have been described. Thus, SF-1-positive cells within the CE show an increase in proliferation during the period when *Sry* is maximally expressed (Schmahl et al., 2000) and mesonephric cells migrate into the gonads and contribute to the peritubular myoid cells and endothelial cells of the vasculature (Capel et al., 1999). Our findings together with those of others allow a model to be proposed that links the action of SRY and SOX9 (see Supplemental Fig. 3). In agreement with studies looking for *Sry* transcripts, we found no expression of either Sry^{Myc} or Sry^{hPLAP} within cells of the CE at any stage. This clearly indicates that SRY does not act within the precursors of the three gonadal cell types, namely, the SF-1-positive cells within the CE. As a result of cell division within the CE, cells enter the genital ridge where they undergo a further asymmetric cell division, where one daughter will become an interstitial cell while the other becomes a supporting cell precursor. The latter may express *Sox9* at a low level. As there was no hPLAP expression in any interstitial cell type, we can conclude that *Sry* expression is initiated only after cells have made the cell-fate decision to become supporting cell precursors. *Sry* begins to be expressed only in these cells, and once its protein product has accumulated above a threshold, *Sox9* expression is up-regulated. This leads not only to *Sry* itself being turned off in that cell, but also to the production of a signal back to the CE to stimulate proliferation and further recruitment of cells into the genital ridge, initiating the cycle over again. Once sufficient, Sertoli cells have begun to differentiate; a further signal permits migration of cells from the underlying mesonephros, which enter the genital ridge and differentiate as endothelial cells and as peritubular myoid cells around the forming Sertoli cell cords. Since overexpression of SOX9 is enough to induce testis differentiation, it can clearly mediate all the downstream events, but a brief pulse of SRY is normally required to set the ball rolling.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2004.07.011.

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